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Characterization of *Schizosaccharomyces pombe* ER α-Mannosidase: A Reevaluation of the Role of the Enzyme on ER-associated Degradation

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It has been postulated that creation of $Man_8GlcNAc_2$ isomer B (M8B) by endoplasmic reticulum (ER) α-mannosidase I constitutes a signal for driving irreparably misfolded glycoproteins to proteasomal degradation. Contrary to a previous report, we were able to detect in vivo (but not in vitro) an extremely feeble ER α-mannosidase activity in *Schizosaccharomyces pombe*. The enzyme yielded M8B on degradation of $Man_9GlcNAc_2$ and was inhibited by kifunensin. Live *S. pombe* cells showed an extremely limited capacity to demannosylate $Man_9GlcNAc_2$ present in misfolded glycoproteins even after a long residence in the ER. In addition, no preferential degradation of M8B-bearing species was detected. Nevertheless, disruption of the α-mannosidase encoding gene almost totally prevented degradation of a misfolded glycoprotein. This and other conflicting reports may be best explained by assuming that the role of ER mannosidase on glycoprotein degradation is independent of its enzymatic activity. The enzyme, behaving as a lectin binding polymannose glycans of varied structures, would belong together with its enzymatically inactive homologue Htm1p/Mnl1p/EDEM, to a transport chain responsible for delivering irreparably misfolded glycoproteins to proteasomes. Kifunensin and 1-deoxymannojirimycin, being mannose homologues, would behave as inhibitors of the ER mannosidase or/and Htm1p/Mnl1p/EDEM putative lectin properties.

INTRODUCTION

Cells have to continuously monitor whether newly synthesized glycoproteins are in the process of proper folding or whether, alternatively, they are irreparably misfolded (Trombetta and Parodi, 2003). Both folding intermediates and irreparably misfolded species may be retained in the endoplasmic reticulum (ER) as a consequence of their association with the ER unconventional chaperones calnexin (CNX) and calreticulin (CRT) (Parodi, 2000). These lectins specifically recognize monoglucosylated *N*-glycans formed either by partial deglucosylation of the transferred glycan (removal of residues *m* and *n* from $Glc_3Man_9GlcNAc_2$; Figure 1) or by UDP-Glc:glycoprotein glucosyltransferase (GT)-mediated reglucosylation of the totally deglucosylated glycan (readdition of Glc *l* to Man *g*; Figure 1). However, as permanent residence of misfolded species in the ER has deleterious effects on cell viability, there are mechanisms for their recognition and subsequent diversion to degradation. It has been proposed that a particular glycan structure ($Man_8GlcNAc_2$ isomer B or M8B; Figure 1) could be the signal by which cells recognize that a glycoprotein molecule is unable to reach its native three-dimensional structure (Knop *et al*., 1996; Jakob *et al*., 1998). Because M8B is produced by ER mannosidase I, a slow-acting enzyme compared with the activity of ER processing glucosidases, it has been speculated that if a protein folds extremely slowly or if it is irreparably misfolded, it would display the particular M8B structure that in turn would be recognized by an ER lectin specific for that glycan and thus diverted to proteasomal degradation. Evidence for the involvement of ER mannosidase I in misfolded glycoprotein degradation was provided by several experimental observations, as for example, that ablation of the *S. cerevisiae* ER mannosidase encoding gene drastically reduced the rate of misfolded glycoprotein degradation and that the same effect resulted from addition of ER mannosidase I inhibitors (kifunensin, KFN or 1-deoxymannojirimycin, DMJ) to mammalian cells (for review, see Cabral *et al*., 2001, and references therein). Concerning the postulated lectin, a presumably enzymatically ER mannosidase I homologue has been identified in *S. cerevisiae* (variously referred to as Htm1p or Mnl1p) and in mammalian cells (referred to as ER degradation enhancing α-mannosidase-like protein or EDEM) (Hosokawa *et al*., 2001; Jakob *et al*., 2001; Nakatsukasa *et al*., 2001). Ablation of Htm1p/Mnl1p encoding gene in yeast or reduction of EDEM expression by the RNA interference technique in mammalian cells drastically reduced disposal of misfolded glycoproteins, whereas overexpression of EDEM increased the degradation rate (Hosokawa *et al*., 2001; Molinari *et al*., 2003; Oda *et al*., 2003). Htm1p/Mnl1p/EDEM has not been isolated yet, so its putative lectin capacity has not been

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Abbreviations used: CNX, calnexin; CRT, calreticulin; CPY, carboxypeptidase Y; CPY*, mutant CPY unable to properly fold; DMJ, 1-deoxymannojirimycin; DTT, dithiothreitol; EDEM, endoplasmic reticulum degradation enhancing α-mannosidase-like protein; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; GT, UDP-Glc:glycoprotein glucosyltransferase; KFN, kifunensin; M8A, M8B, and M8C, $Man_8GlcNAc_2$ isomers as defined in Figure 1; MG132, *N*-carboxybenzoxyl-leucinyl-leucinyl-leucinal; NMDNJ, *N*-methyldeoxynojirimycin.

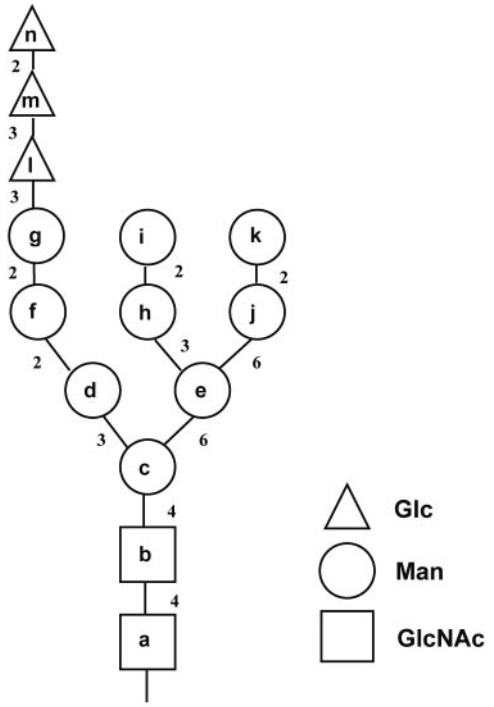

Figure 1. Glycan structures. The figure represents the glycan transferred to proteins in wild-type *S. pombe* cells ($Glc_3Man_9GlcNAc_2$). Lettering corresponds to the order of residue addition in the synthesis of the dolichol-P-P derivative. Numbers between monosaccharides correspond to the carbon atoms involved in the respective linkages. M8A lacks residues *g* and *l-n*, M8B residues *j* and *l-n*, and M8C residues *k-n*. The $Man_7GlcNAc_2$ isomer formed in *S. pombe* lacks residues *i* and *k-n*. The glycan transferred in MadIA214cells ($Man_5GlcNAc_2$) lacks residues *h-n*.

confirmed. The human genome codes for two additional EDEM-like proteins. Characterization of the so called EDEM2 revealed that it displays no mannosidase activity and that its overexpression in mammalian cells enhances misfolded glycoprotein degradation (Mast *et al*., 2005; Olivari *et al*., 2005). Both the *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* genomes code for only one Htm1p/Mnl1p/EDEM protein. On the other hand, in *S. cerevisiae* there is an alternative, proteasome-independent pathway for ER-associated degradation (ERAD) (Umebayashi *et al*., 2001).

The model by which *N*-glycan structural modifications introduced by ER α-mannosidase activity could play an essential role in driving irreparably misfolded glycoproteins to degradation is extremely attractive but presents several severe inconsistencies that will be discussed below in extenso. To further study the controversial role of ER mannosidase on ERAD of misfolded glycoproteins, we chose the fission yeast *S. pombe* because this yeast, contrary to *S. cerevisiae*, displays a quality control of glycoprotein folding similar to that present in mammalian cells. The fission yeast expresses a robust GT activity responsible for the glucosylation of folding intermediates and irreparably misfolded species (Fernández *et al*., 1994). *S. pombe* GT is up-regulated under conditions of ER stress and ablation of its encoding gene triggers the so-called unfolded protein response (Fernández *et al*., 1996; D'Alessio *et al*., 1999). In addition, GT was shown to be essential for cell viability under conditions of severe ER stress (Fanchiotti *et al*., 1998). What made *S. pombe* a particularly interesting model system for the purpose of our work is that according to a previous report, no ER mannosidase activity could be detected in it, both in in vivo and in vitro assays (Ziegler *et al*., 1994). Work here reported shows that this yeast displays indeed an extremely feeble ER mannosidase activity that could be detected in vivo but not in vitro. The extremely limited capacity to demannosylate $Man_9GlcNAc_2$ glycans present in misfolded glycoproteins severely questions the accepted role of ER α-mannosidases on ERAD.

MATERIALS AND METHODS

Materials

[^{14}C]Glc (301 Ci/mol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). *N*-Methyldeoxynojirimycin (NMDNJ), KFN, and DMJ were from Toronto Biochemicals Toronto, Ontario, Canada. *N*-Carboxybenzoxyl-leucinyl-leucinyl-leucinal (MG132) and *clasto*-lactacystin-β-lactone (lactacystin) were from Calbiochem (San Diego, CA). Restriction enzymes and other enzymes used in DNA procedures were from PerkinElmer Life and Analytical Sciences. Dithiothreitol (DTT) and endo-β-*N*-acetylglucosaminidase H (Endo H) were from Sigma-Aldrich (St. Louis, MO).

Strains and Culture Media

The *S. pombe* strain was ADp (*h*$^+$, *ade6*, *M216*, *leu1-32*, *ura4-D18*). Growth medium contained 0.5% yeast extract (Difco, Detroit, MI), 3% glucose, and 75 mg/ml adenine. Minimal medium was as described previously (Alfa *et al*., 1993). The *S. cerevisiae* strain used was HH3 (*MAT*α, *lys2-801*, *ade2-101*, *his3-*Δ*200*, *trp1-1*, *ura3-52*, and *leu2-*Δ*1*).

Antisera. *S. cerevisiae* caboxypeptidase Y (CPY) and *S. pombe* CNX antisera were generous gifts from Drs. Reid Gilmore (University of Massachusetts, Worcester, MA) and Luis Rokeach (Université de Montréal, Montréal, Quebec, Canada), respectively.

Labeling, Isolation, and Structural Analysis of N-Glycans

Cells were labeled and *N*-glycans isolated as described previously (Fernández *et al*., 1994). Briefly, cells (0.3 g) were resuspended in 1 ml (total volume) of 1% yeast nitrogen base (YNB; Difco) to which 100 μl of 50 mM Glc containing 150 μCi of [^{14}C]Glc was added. Incubation times were as described in text. Where indicated 100 μl of 0.5 M Glc was added for chasing the label. KFN, DMJ, and NMDNJ (2.5 mM final concentrations) and lactacystin (50 μM final concentration) were added 30 min and DTT (5 mM final concentration) 5 min before labeling. In experiments described in Figure 7, A–C, cycloheximide (0.1 mg/ml final concentration) together with Glc (50 mM final concentration) were added 15 min after addition of the label and DTT concentration was raised to 10 mM after 35 min of chase. In experiment described in Figure 7D, DTT (5 mM final concentration) was added 70 min before the label and the drug concentration was raised to 10 mM 5 min before [^{14}C]Glc addition. Acetolysis and paper electrophoresis in 0.1 M sodium molybdate, pH 5.0, were as described previously (Engel and Parodi, 1985). Chromatography was performed on Whatman no. 1 paper with solvents A, 1-propanol/nitromethane/water (5:2:4); B, 1-butanol/pyridine/water (4:3:4); C, 1-butanol/pyridine/water (10:3:3); and D, 2-propanol/acetic acid/water (29:4:9).

Gene Disruptions

***Disruption of* spmns1$^+$.** A 1390-base pair fragment of gene *spmns1+* (gi: 6322591) was amplified using genomic DNA as template and primers 5′-TAACCGCGGATGGTTAAACGAAGGACTG-3′ and 5′-CAACTCGAGCTTCGACTCTACACTATC-3′ and cloned in the pBluescript KSII(+) vector. Fragment *Xba*I-B*gl*II corresponding to bases 550–816 was replaced with gene *ura4*$^+$. A 2946-base pair fragment liberated with *Sac*II and *Xho*I was used to transform *S. pombe* cells. Transformants were isolated in minimal medium supplemented with adenine and leucine. Correct integration was checked by Southern blotting analysis and by PCR with a primer from *ura4*$^+$ (5′-TTTTCATCCCCTCAGCTC-3′) and from *spmns1*$^+$ (5′-TAACTCGAGCACCGTGTACAGGTCC-3′). Disruption of *spmns2*$^+$ and s*pmnl1*$^+$. The same procedures were used for disruption of these genes, but in the first case amplification primers were 5′-GGACCTGCAGATGACACTCTTCCCAGTA-3′ and 5′-CTTCTCGAGGGCTCTCCAACTAGTTGG-3′ (2985-base pair fragment) and in the second 5′-TATATACCGCGGATGGGTAGCTTGCACTC-3′ and 5′-CAACTCGAGTTCACCATCCGTGATG-3′ (2205 base pairs fragment). In the first case, a *Hin*dII fragment corresponding to bases 428-2324 and in the second a *Cla*I-*Xba*I fragment corresponding to bases 863-1600 was replaced with gene *ura4*$^+$. In the first case, a 2946-base pair fragment liberated with *Pst*I and *Xho*I and in the second a 3227-base pair fragment liberated with *Sac*II and *Xho*I were used to transform *S. pombe* cells. Correct integration was checked

by Southern blotting analysis and by PCR using primers from *ura4*$^+$ 5′-TTTTCATCCCCTCAGCTC-3′ in the first case and 5′-TGCTCCTACAACATTACC-3′ in the second and primers from *spmns2*$^+$ 5′-TAACTCGAGCACCGTGTACAGGTCC-3′ and from *spmnl1*$^+$ 5′-TTGACGCACAGAGACGTTG-3′.

Sequence Analysis

The program used for calculating similarities between sequences was the Water from the European Bioinformatics Institute (www.ebi.ac.uk) using BLOSUM62 as matrix.

In Vitro α-Mannosidase Assays

S. cerevisiae and *S. pombe* microsomes were prepared as described previously (Fernández *et al*., 1994). One milligram of microsomal proteins was incubated with 7000 cpm of [^{14}C-*Man*]Man$_9$GlcNAc in 50 mM PIPES buffer, pH 7.1, 1 mM $CaCl_2$ and 0.4% Lubrol in a total volume of 100 μl. After 30 min at room temperature, 200 ml of methanol was added, tubes were centrifuged at low speed, and supernatants were spotted on Whatman no. 1 paper. Chromatograms were developed with solvent D.

S. cerevisiae CPY Expression in S. pombe*

S. cerevisiae gene encoding for caboxypeptidase Y (CPY) was amplified using *Pfu* polymerase, genomic DNA as template and primers 5′-CGTCTCGAGATGAAAGCATTCACC-3′ and 5′-ATACCCGGGTTATAAGGAGAAACCACCGTG-3′ and cloned in vector pBluescript KS II (+). Base 763 was mutated (G to A) by PCR with primers 5′-CATCGCT**A**GGGAATCCTAC-3′ and 5′-TGGAAATCTTGGCCCTTG-3′. The product (encoding CPY*) was sequenced to check the mutation introduced and cloned in sites *Xho*I and *Sma*I of *S. pombe* expression vector pREP3X, which was used to transform wild-type and mutant *S. pombe* strains as described previously (Fanchiotti *et al*., 1998).

CPY degradation in S. pombe*

Cells expressing *S. cerevisiae* CPY* were grown in SC medium (0.67% YNB [Difco], 2% Glc, and 70 mg/ml adenine and uracyl) at 28°C up to an OD_{600} of 0.2–0.3. About 15 OD_{600} of cells was withdrawn, centrifuged, and resuspended in 0.6 ml of SC medium but without Met (YNB without amino acids; Difco). Cells were incubated for 30 min, and 300 μCi of EasyTaq Express Labeling Mix (PerkinElmer Life and Analytical Sciences) was added. Where indicated, KFN, DMJ, lactacystin, and MG132 (final concentrations, 2.5 mM the first two drugs and 50 μM the last two) were added 30 min before the label. Cycloheximide (1 mM final concentration) was added 15 min after the label, and 200-μl aliquots were withdrawn 0 and 30 min after addition of the protein synthesis inhibitor. Two hundred milliliters of 2× stop buffer (2 M sorbitol, 50 mM Tris-HCl, pH 7.5, 40 mM NaN_3, and 20 mM DTT) was added to the samples, which were then frozen to –70°C until extract preparation. For this purpose 5 μl of lysing enzymes (10 mg/ml) was added, and samples were incubated for 25 min at 30°C. Trichloroacetic acid (5% final concentration) was added and samples were maintained on ice for 30 min. Samples were then centrifuged, and pellets were washed with ice-cold acetone. Pellets were resuspended in 100 μl of boiling buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% SDS). Resuspended samples were sonicated, stirred in a Vortex with glass beads and boiled for 4 min. Immunoprecipitation buffer (1 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween 20, and 0.1 mM EDTA) was then added. The extracts were centrifuged and to supernatants CPY antiserum was added. Samples were then incubated overnight at 4°C with gentle stirring. Protein A-Sepharose was then added, samples were incubated for 3 h and then centrifuged at low speed. Supernatants were withdrawn and CNX antiserum was added to them. Pellets of the anti-CPY immunoprecipitations were washed first with immunoprecipitation buffer and then with the same solution but containing in addition 2 M urea, 0.1% SDS, and finally with Tris buffer saline. Pellets were then heated at 100°C for 5 min with 2× sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, and 6% SDS). Supernatants were run on SDS-PAGE, dried, and CPY* quantified with a PhosphoImager. Immunoprecipitations with CNX antiserum were likewise performed. Amounts of CNX occurring under different conditions were used to normalize recovery of CPY* in the samples.

RESULTS

S. pombe Expresses an α-Mannosidase

As mentioned above, a previous report indicated that *S. pombe* cells do not express an ER mannosidase (Ziegler *et al*., 1994). Nevertheless, incubation of live wild-type *S. pombe* cells with 5 mM radioactive Glc for 15 min led to formation of Endo H-released protein-linked Man$_9$GlcNAc and Man$_8$GlcNAc. The proportion of the smaller compound ranged between 5 and 8% (Figure 2A). Practically no larger glycans were observed after a 15-min incubation but Golgi elongation of above-mentioned *N*-glycans was observed on chasing cells with a 10-fold higher unlabeled Glc concentration (Figure 2B). Man$_8$GlcNAc$_2$ was also formed when cells were labeled at 50 mM Glc concentration.

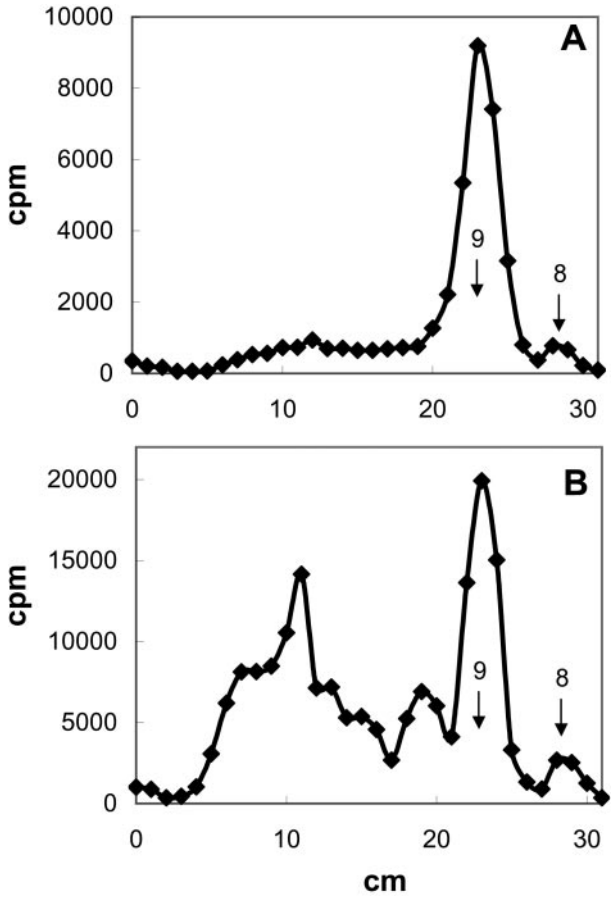

Figure 2. *S. pombe* expresses an α-mannosidase activity. (A) *S. pombe* cells were incubated for 15 min with 5.0 mM labeled Glc, and whole cell Endo H-released glycans were run on paper chromatography with solvent A. (B) The same as in A, but cells were chased with 50 mM Glc for 45 min. Standards: 9, Man$_9$GlcNAc; and 8, Man$_8$GlcNAc. For further details, see *Materials and Methods*.

Identification of the α-Mannosidase Encoding Gene

There are three proteins encoded in the *S. pombe* genome with potential α-mannosidase activity: Spmns1p (gi48474992), Spmns2p (gi:19114891), and Spmnl1p (gi:19115346) (Sp stands for *S. pombe*). They, respectively, show higher similarity to *S. cerevisiae* ER α-mannosidase (56.8%), to vacuolar mannosidase (65.1%), and to the mannosidase-like protein (otherwise referred to as Htm1p/Mnl1p/EDEM) (45.0%).

Their encoding genes were individually disrupted and resulting mutants assayed for formation of protein-linked glycans. Only the mutant lacking Spmns1p showed no formation of Man$_8$GlcNAc$_2$ (Figure 3, A–D). Transcription of the Htm1p/Mnl1p/EDEM-encoding gene (*spmnl1*$^+$) in wild-type cells was confirmed by reverse transcription-PCR.

Structural Characterization of Man$_8$GlcNAc$_2$

Man$_8$GlcNAc was submitted to acetolysis, a procedure that preferentially cleaves α(1,6) bonds between Man units, and resulting fragments were separated on paper chromatogra-

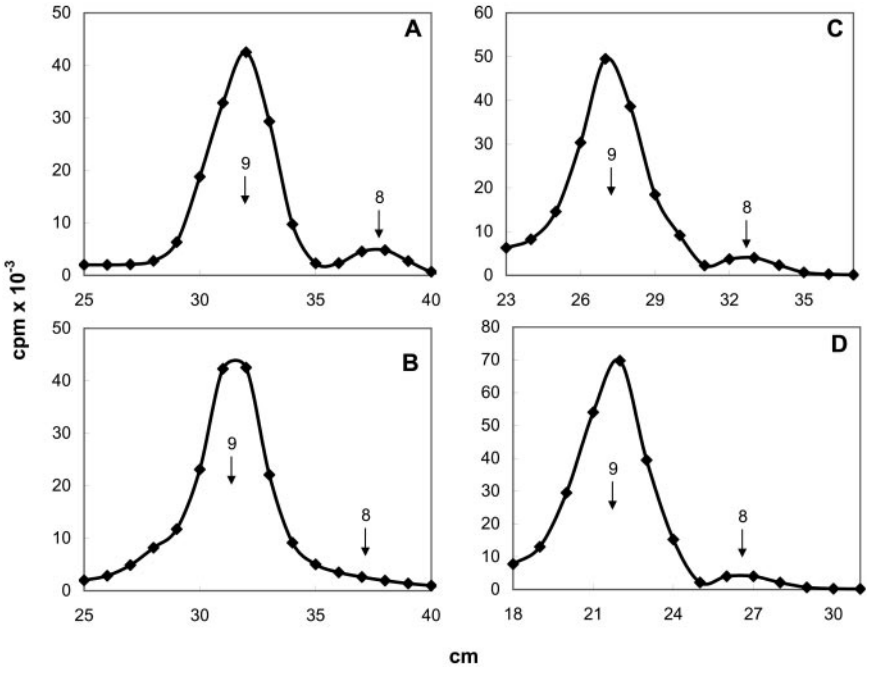

Figure 3. Synthesis of M8B in wild-type and mutant *S. pombe* cells. Wild type (A), *spmns1*$^{-}$ (B), *spmns2*$^{-}$ (C), and *spmnl1*$^{-}$ (D) mutant cells were labeled for 15 min with 5 mM labeled Glc, and whole cell, Endo H-sensitive glycans were run on paper chromatography with solvent A. Standards: 9, $Man_9GlcNAc$; and 8, $Man_8GlcNAc$. For further details, see *Materials and Methods*.

phy. Labeled compounds migrated as $Man_4GlcNAc$ and Man_2 standards (Figure 4A). Reduction of the latter followed by paper electrophoresis in sodium molybdate, pH 5.0, revealed that about half of the label remained at the origin, whereas the rest migrated to the anode (Figure 4B). As α(1,2)-linked reduced Man_2 migrates under the assay conditions, whereas the α(1,3)-linked isomer does not, results obtained indicate that $Man_8GlcNAc_2$ isomer B (M8B) was produced by *S. pombe* Spmns1p mannosidase (Figure 1) (Parodi *et al.*, 1983). A mixture of M8B and M8C isomers (Figure 1) was obtained if the Glc concentration during the labeling period of wild-type cells was reduced to 0.5 mM. On the other hand, the Spmns1p minus mutant only yielded M8C under similar labeling conditions. Because M8C but not M8B is an intermediate in the synthesis of dolichol derivatives, these results indicate that under low Glc concentrations, *S. pombe* cells, the same as the mammalian cells, synthesize and transfer to proteins a sizable proportion of incomplete glycans (Gershman and Robbins, 1981; Rearick *et al.*, 1981).

The Effect of α-Mannosidase Inhibitors

KFN and DMJ were tested as potential Spmns1p inhibitors. Wild-type cells were preincubated with the compounds at a

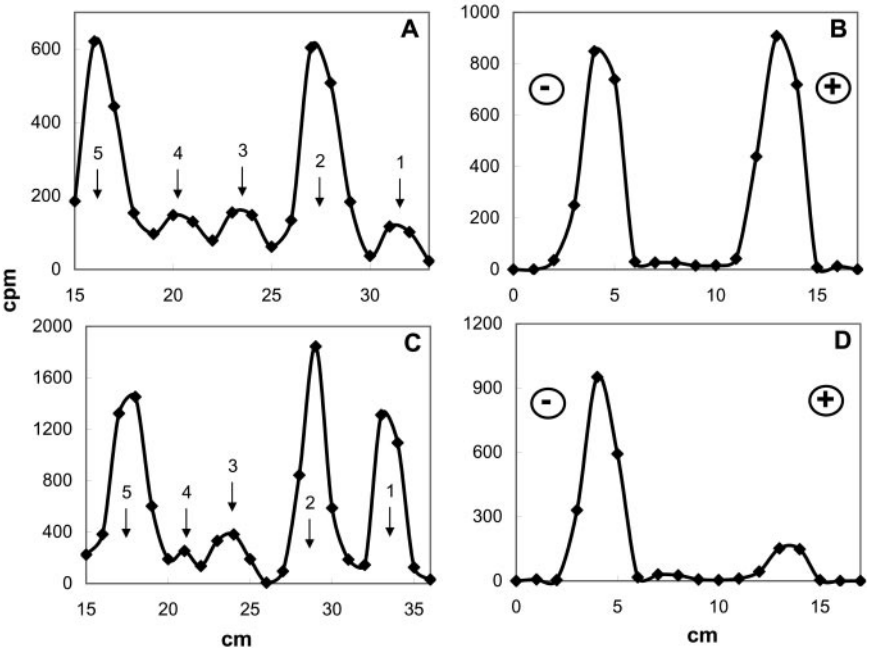

Figure 4. Structures of glycans. (A) $Man_8GlcNAc$ synthesized by wild-type *S. pombe* cells was submitted to acetolysis and run on paper chromatography with solvent B. (B) Man_2 in A was reduced with $NaBH_4$ and run on paper electrophoresis with 0.1 M sodium molybdate, pH 5.0. (C) Paper chromatography of acetolysis products of $Man_7GlcNAc$. (D) Paper electrophoresis of reduced Man_2 isolated from chromatogram shown in Figure 4C. Standards: 1, Man; 2, Man_2, 3, Man_3, 4, $Man_3GlcNAc$; and 5, $Man_4GlcNAc$. For further details, see *Materials and Methods*.

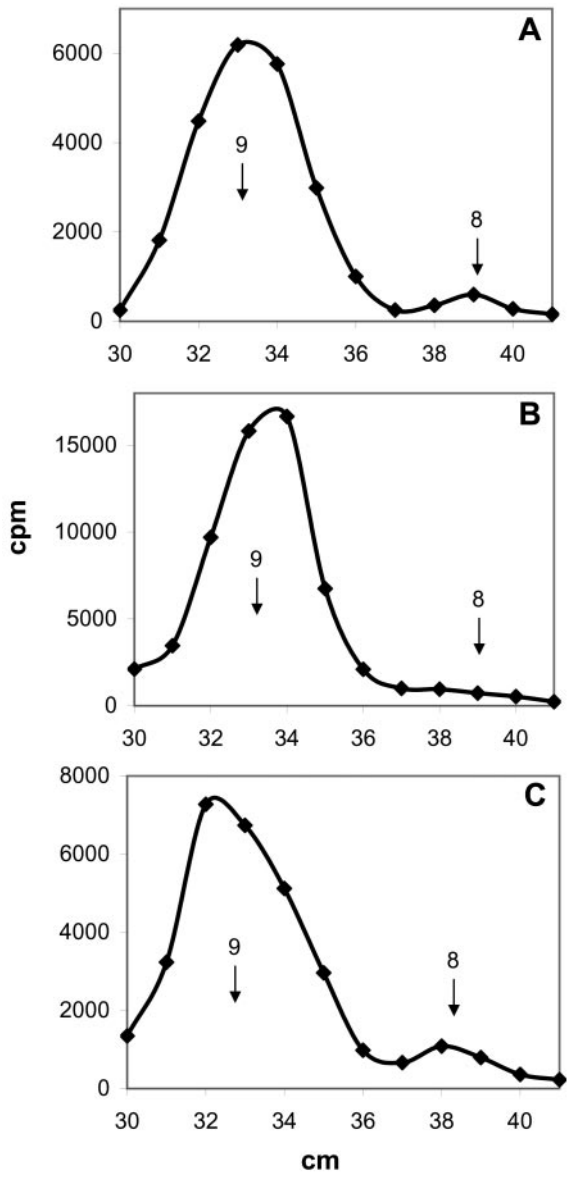

Figure 5. Effect of α-mannosidase inhibitors on *S. pombe* α-mannosidase (Spmns1p). (A) *S. pombe* wild-type cells were incubated for 15 min with 5 mM labeled Glc, and whole cell Endo H-released glycans were run on paper chromatography with solvent A. In B and C, 2.5 mM (final concentration) of KFN or DMJ, respectively, were added 30 min before the label. Standards: 9, $Man_9GlcNAc$; and 8, $Man_8GlcNAc$. For further details, see *Materials and Methods*.

2.5 mM concentration for 30 min before addition of labeled glucose. As depicted in Figure 5, A–C, whereas KFN significantly inhibited M8B formation, absolutely no effect was observed upon addition of DMJ. No inhibition was observed also when 2.5 mM DMJ was tested under similar in vivo conditions as *S. cerevisiae* ER α-mannosidase inhibitor. Because the DMJ concentration used is 50-fold higher than the IC_{50} of the drug for the last enzyme as determined in cell free assays (Jelinek-Kelly *et al*., 1985), this indicates that penetration of DMJ into the budding yeast ER, and probably also into that of *S. pombe*, is severely impeded.

Subcellular Localization of Spmns1p

Wild-type cells were incubated with 5 mM labeled glucose for 15 min in the presence of DTT. This compound effectively prevents proper folding, and thus ER exit, of glycoproteins by interfering with disulfide bond formation (Simons *et al*., 1995). Secretion of disulfide-free proteins is not affected, but as already reported and as will be further shown below, the majority of glycoproteins synthesized in *S. pombe* have disulfide bonds (Fernández *et al*., 1998). M8B was still formed under the experimental conditions used, thus indicating that Spmns1p localized to the ER (Figure 6A). To confirm the subcellular localization of *S. pombe* α-mannosidase, we took advantage of the presence of GT in this yeast ER: an *alg6* mutant was incubated for 15 min with labeled glucose in the presence of 5 mM DTT and 2.5 mM NMDNJ, a glucosidase II inhibitor. In *alg6* mutants, $Man_9GlcNAc_2$ instead of $Glc_3Man_9GlcNAc_2$ is transferred to proteins because they lack the enzyme that transfers the first Glc residue from Glc-P-dolichol to $Man_9GlcNAc_2$-P-P-dolichol. Compounds migrating in the positions expected for $Glc_1Man_9GlcNAc$, $Man_9GlcNAc$, and $Glc_1Man_8GlcNAc$ were formed (Figure 6B). Rather surprisingly, no $Man_8GlcNAc$ occurred in the chromatogram. Compounds migrating as $Glc_1Man_9GlcNAc$, $Man_9GlcNAc$ and $Glc_1Man_8GlcNAc$ were individually rerun on paper chromatography to get a better separation and submitted then to strong acid hydrolysis followed by monosaccharide separation by paper chromatography. As shown in Figure 6, C–E, compounds migrating as $Glc_1Man_9GlcNAc$ and $Glc_1Man_8GlcNAc$ contained labeled Glc and Man units, whereas that migrating as $Man_9GlcNAc$ only contained the last residues. Formation of $Glc_1Man_8GlcNAc_2$ indicated that *S. pombe* α-mannosidase (Spmns1p) and GT shared the same subcellular compartment.

Glycan Processing in Misfolded Glycoproteins

Figure 2, A and B, shows that a minimal proportion of *N*-glycans was processed to $Man_8GlcNAc_2$ under normal folding conditions. To study ER processing of *N*-glycans present in misfolded species, *S. pombe* cells were incubated with 5 mM [^{14}C]Glc for 15 min in the presence of DTT and lactacystin to prevent protein folding and degradation, respectively. Contrary to what happens in *S. cerevisiae*, *S. pombe* wild-type cells are freely permeable to the proteasome inhibitor. Cells were then chased with 50 mM Glc, and 0.15 mg/ml cycloheximide was added to hinder further protein synthesis and thus *N*-glycosylation. Samples were withdrawn 0, 15, 45, and 90 min after the chase. The pattern of the *N*-glycans of the 0-min chase sample was similar to that shown in Figure 2A, whether incubation had been performed in the presence or absence of DTT. The sample obtained after 45-min chase in presence of the drug showed little if any Golgi elongation of *N*-glycans (Figure 7A) and the presence of both $Man_8GlcNAc$ and $Man_7GlcNAc$ in addition to $Man_9GlcNAc$. No $Man_6GlcNAc$ was detected. The pattern yielded by cells pulse chased in the absence of DTT revealed a normal Golgi processing of glycans (Figure 7B). This showed that DTT had effectively prevented ER exit of most glycoproteins. The relative proportions of $Man_9GlcNAc$, $Man_8GlcNAc$, and $Man_7GlcNAc$ in the samples are represented in Figure 7C. It may be observed that the rate of $Man_9GlcNAc_2$ processing gradually decreased and finally completely ceased although, as shown in Figure 7, A and B, glycoproteins were still in the ER after the 45-min chase as they did not show a significant Golgi processing. Moreover, DTT inactivation of ER α-mannosidase was ruled out by preincubating cells for 70 min in the presence of the

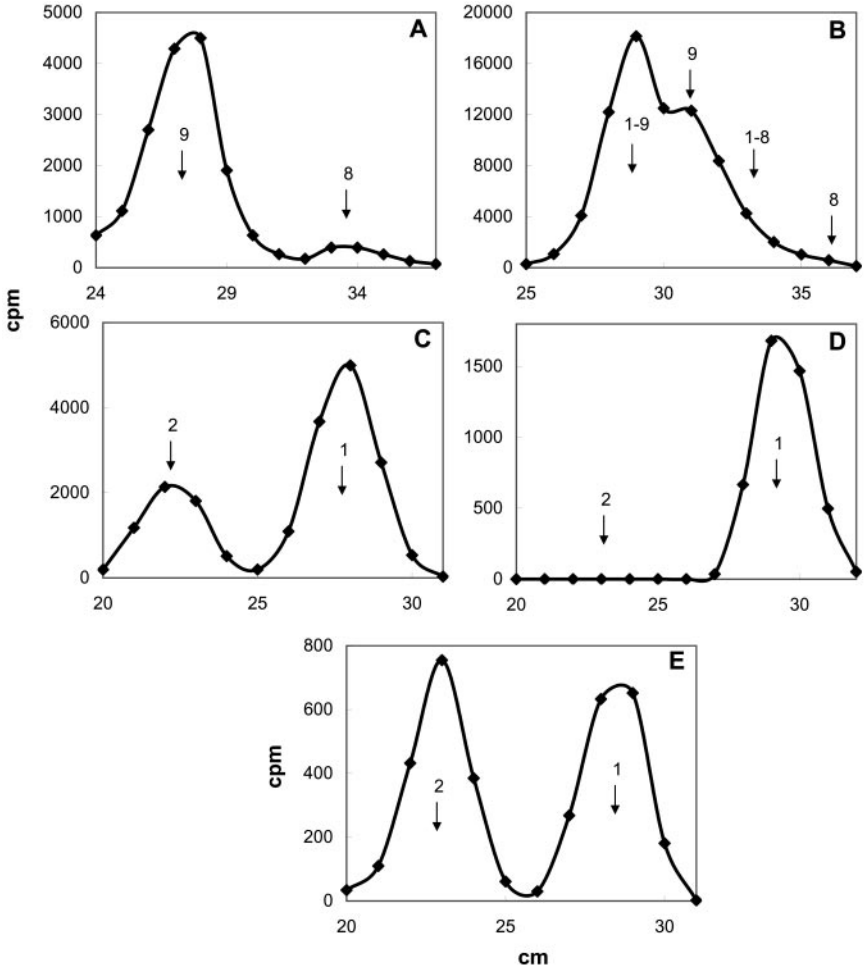

Figure 6. Subcellular localization of *S. pombe* α-mannosidase (Spmns1p). (A) *S. pombe* wild-type cells were incubated for 15 min with 5 mM labeled Glc, and whole cell Endo H-released glycans were run on paper chromatography with solvent A. DTT (5 mM final concentration) was added 5 min before the label. (B) *S. pombe alg6* mutant were preincubated with 2.5 mM NMDNJ for 30 min and 5 mM DTT for 5 min and incubated for 15 min with 5 mM labeled Glc. Whole cell Endo H-released glycans were run on paper chromatography with solvent A. Material running between 27 and 29 cm ($Glc_1Man_9GlcNAc$), 31 and 32 cm ($Man_9GlcNAc$), and 33 and 35 ($Glc_1Man_8GlcNAc$) in B was eluted and run for a second time on paper chromatography with solvent A. Material running as $Glc_1Man_9GlcNAc$ (C), as $Man_9GlcNAc$ (D), and as $Glc_1Man_8GlcNAc$ (E) was submitted to strong acid hydrolysis (1 N HCl for 4 h at 100°C) and run on paper chromatography with solvent C. Standards: 1–9, $Glc_1Man_9GlcNAc$; 9, $Man_9GlcNAc$; 1–8, $Glc_1Man_8GlcNAc$; 8, $Man_8GlcNAc$; 2, Glc; and 1, Man. For further details, see *Materials and Methods*.

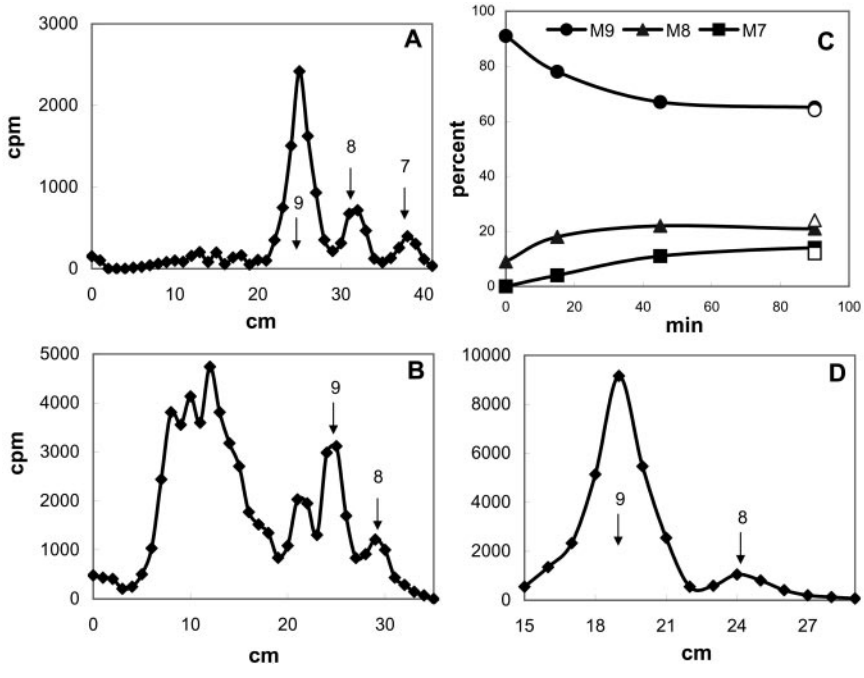

Figure 7. *S. pombe* ER processing of glycans in misfolded glycoproteins. *S. pombe* wild-type cells were preincubated with lactacystin (50 μM final concentration) for 30 min and DTT (5 mM final concentration) for 5 min and incubated with 5 mM labeled Glc for 15 min. Glc concentration was then raised to 50 mM with unlabeled Glc and cycloheximide (0.15 mg/ml final concentration) was added. Samples were withdrawn 0, 15, 45, and 90 min after the chase. DTT concentration was raised to 10 mM 35 min after the chase. (A) Whole cell glycans Endo H-released from the 45-min chase sample. (B) Same as A, but cells were incubated in the absence of DTT. (C) Proportion of $Man_9GlcNAc$ (full circles), $Man_8GlcNAc$ (full triangles), and $Man_7GlcNAc$ (full squares) in the samples. Data in empty symbols correspond to a sample incubated in the absence of lactacystin. (D) Cells were incubated with DTT (5 mM final concentration) for 65 min, the drug concentration was then raised to 10 mM and 5 min later 5 mM labeled Glc was added. Incubation lasted for 15 min. Whole cell Endo H-released glycans were run with solvent A. Standards: 9, $Man_9GlcNAc$; and 8, $Man_8GlcNAc$. For further details, see *Materials and Methods*.

drug followed by a 15-min pulse with [^{14}C]Glc (Figure 7D). The most plausible explanation for results shown in Figure 7C is that misfolded glycoproteins had migrated to an ER domain lacking α-mannosidase activity. Cells pulsed chased in the presence or absence of lactacystin yielded almost identical proportions of $Man_9GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_7GlcNAc_2$ after the 90-min chase (Figure 7C). Because lactacystin but not DTT hindered proteasomal degradation and substantial degradation of glycoproteins unable to properly fold is expected to had occurred in the absence of the former drug after a 90-min chase (see below), this result indicates that no preferential degradation of misfolded glycoprotein-linked M8B over $Man_9GlcNAc_2$ or $Man_7GlcNAc_2$ had occurred.

Structural characterization of $Man_7GlcNAc$ was performed as described above for $Man_8GlcNAc$. Acetolysis of $Man_7GlcNAc$ yielded $Man_4GlcNAc_2$, Man_2, and Man (Figure 4C). Further reduction of the disaccharide followed by paper electrophoresis in 0.1 M sodium molybdate, pH 5.0, revealed that the compound was Manα(1,3)Man (Figure 4D). The structure of $Man_7GlcNAc$ is depicted in Figure 1. *S. pombe* ER α-mannosidase seemed to have, therefore, the same specificity as the *S. cerevisiae* enzyme (Herscovics *et al*., 2002). From results here presented it may be concluded that although the fission yeast had an extremely limited capacity to demannosylate *N*-glycans in misfolded glycoproteins, both M8B and $Man_7GlcNAc_2$ were produced on ER processing of glycans. Moreover, even this last compound, which was the smallest yielded by ER processing, displayed the acceptor Man unit involved in GT-mediated reglucosylation (residue *g*, Figure 1).

In Vitro Assay of S. pombe *ER α-Mannosidase: Comparison with S. cerevisiae*

S. pombe and *S. cerevisiae* microsomal membranes were incubated with [^{14}C-*Man*]$Man_9GlcNAc$ under identical conditions, the reactions stopped with 66% methanol, and the supernatants run on paper chromatography. Only *S. cerevisiae* microsomes produced Man (Figure 8A). Although the proportion of label in the monosaccharide (~12%) indicated that the reaction had reached completion in the case of the budding yeast, absolutely no labeled monosaccharide was produced by *S. pombe* microsomes. Mixed membrane experiments discarded the presence of an inhibitor or a strong protease in the fission yeast microsomes. Moreover, the same result was obtained when a strong antiproteolytic cocktail was used for *S. pombe* microsome preparation. A previous report also communicated that in vitro assays had failed to detect *S. pombe* ER α-mannosidase (Ziegler *et al*., 1994). To further compare *S. cerevisiae* and *S. pombe* ER α-mannosidase activities, we incubated the former cells with labeled [^{14}C]Glc under conditions identical to those described in Figure 2A for the latter cells, but for 5 min instead of 15 min. As depicted in Figure 8B, the pattern of *N*-glycans was composed of ~80% $Man_8GlcNAc$ and almost equal proportions of $Man_9GlcNAc$ and $Man_7GlcNAc$. *S. cerevisiae* had, therefore, at least more than one order of magnitude higher capacity than *S. pombe* to process *N*-linked $Man_9GlcNAc_2$.

CPY Degradation in S. pombe*

The extremely feeble ER α-mannosidase activity present in *S. pombe*, that even after 90 min could not degrade >35% of $Man_9GlcNAc_2$ present in misfolded glycoproteins, suggested that the enzyme might not be related to ERAD in the fission yeast. To test this possibility, we expressed in *S. pombe* a mutant of *S. cerevisiae* carboxypeptidase Y unable to properly fold (CPY*). Cells were pulsed for 30 min with [^{35}S]Met+Cys and chased. CPY* degradation was followed by immunoprecipitation, SDS-PAGE, and autoradiography. As depicted in Figure 9, ~61% of CPY* had disappeared from wild-type cells after a 30-min chase. A similar value has been reported for degradation of the same glycoprotein in *S. cerevisiae* after the same chase period (Jakob *et al*., 1998). A sharply decreased degradation was observed when CPY* was expressed in Spmns1p or Spmnl1p minus mutants, the same as has been described for *S. cerevisiae* (Figure 9). These results showed that indeed the presence of ER α-mannosidase and of the α-mannosidase-like protein (Htm1p/Mnl1p/EDEM) was fundamental for misfolded glycoprotein degradation also in *S. pombe*. CPY* degradation was totally or almost totally prevented in the presence of KFN, an effective ER α-mannosidase inhibitor, or of the proteasomal inhibitors lactacystin or MG132 (Figure 9). Neither DMJ nor DTT modified the extent of degradation of CPY* expressed in wild-type cells. The lack of effect of the former drug was expected, because it did not inhibit in vivo

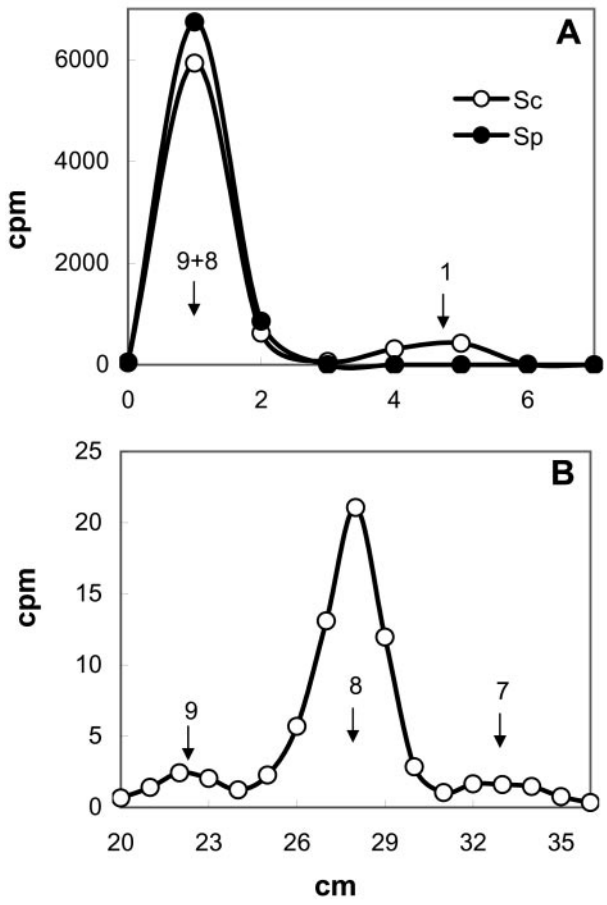

Figure 8. In vitro assay of *S. pombe* ER α-mannosidase. Comparison with *S. cerevisiae*. (A) *S. pombe* (full circles) and *S. cerevisiae* (empty circles) microsomes were incubated with [^{14}C-Man]$Man_9GlcNAc$, two volumes of methanol were added and the supernatants run on paper chromatography with solvent D. (B) *S. cerevisiae* cells were incubated for 5 min with 5 mM labeled Glc, and whole cell Endo H-released glycans were run on paper chromatography with solvent A. Standards: 9, $Man_9GlcNAc$; 8, $Man_8GlcNAc$; 7, $Man_7GlcNAc$; and 1, Man. For further details, see *Materials and Methods*.

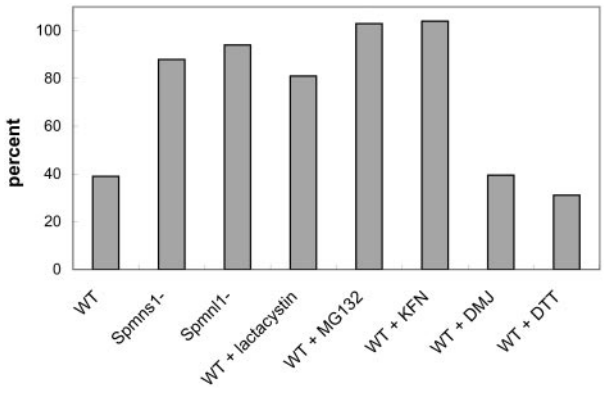

Figure 9. CPY* degradation in *S. pombe*. Wild-type or indicated mutant *S. pombe* cells expressing *S. cerevisiae* CPY* were labeled for 30 min with [^{35}S]Met+Cys. Protein synthesis was stopped upon addition of cycloheximide, and aliquots were withdrawn 0 and 30 min after addition of the protein synthesis inhibitor. Cells were lysed and CPY* immunoprecipitated, run on SDS-PAGE, and submitted to autoradiography. KFN, DMJ, lactacystin, and MG132 (final concentrations: 2.5 mM the first two drugs and 50 μM the last two drugs) were added 30 min and DTT (5 mM) 5 min before the label. Recovery of CPY* in immunoprecipitates was normalized by quantification of immunoprecipitated CNX from the same extracts.

Spmns1p activity, probably because an impediment in the penetration into the ER lumen (Figure 5C).

DISCUSSION

Here, we show that, contrary to a previous report (Ziegler *et al*., 1994), *S. pombe* displays an α-mannosidase that localizes to the ER and we have identified its encoding gene. The enzyme seemed to be inhibited by KFN and to display the same specificity as its homologue from *S. cerevisiae* because it also produced first M8B and then the same $Man_7GlcNAc_2$ isomer upon $Man_9GlcNAc$ degradation.

The most remarkable feature of *S. pombe* ER α-mannosidase is the feeble activity that it displays in vivo and our and others inability to detect its activity in in vitro assays (Ziegler *et al*., 1994). Triggering misfolding of newly synthesized glycoproteins by DTT addition resulted in hindering their exit from the ER and in a limited conversion of $Man_9GlcNAc_2$ to M8B and $Man_7GlcNAc_2$. After an initial burst of $Man_9GlcNAc_2$ degradation, all activity on misfolded glycoproteins ceased probably due to migration of the latter to ER domains lacking α-mannosidase. This result suggests that M8B might not be a signal for diversion of misfolded glycoproteins to degradation in *S. pombe*. Although in this yeast, the same as in other cells, the proportion of misfolded glycoproteins that are degraded through the glycan- and proteasome-dependent and proteasome-independent ERAD pathways is unknown, it should be expected that even if the former represented a minor pathway compared with the independent pathway, degradation of $Man_9GlcNAc_2$ linked to misfolded glycoproteins to $Man_8GlcNAc_2$ should have been fully completed after 90 min and not stopped at a 35% level after 45 min. An exclusive demannosylation of glycoproteins following the dependent pathway (i.e., bearing the 35% of glycans degraded) is highly unlikely because no ER mannosidase able to distinguish between glycoproteins that follow the alternative degradation pathways has been described so far. In fact, all ER α-mannosidases characterized already have been shown not to sense the conformational or soluble or membrane-bound status of the protein moieties. Exactly the same proportion of $Man_9GlcNAc_2$, M8B, and $Man_7GlcNAc_2$ glycans was produced under conditions allowing or not glycoprotein proteasomal degradation. These results also suggest that M8B might not be a signal for diverting misfolded glycoproteins to degradation, although this conclusion is valid only if the glycan- and proteasome-dependent ERAD pathway represents a relatively major pathway. *S. pombe* had a much lower capacity to process $Man_9GlcNAc$ than *S. cerevisiae*: in vitro assays in which all the labeled glycan had been converted to M8B with microsomes prepared from the budding yeast failed to release any detectable radioactivity when *S. pombe* membranes were used. Moreover, >90% of $Man_9GlcNAc_2$ had been processed to M8B and $Man_7GlcNAc_2$ after a 5-min incubation of S. *cerevisiae* cells with labeled glucose, whereas a 15-min incubation of *S. pombe* cells only showed 5–8% of glycan conversion. It is unknown for the moment whether the extremely low α-mannosidase activity observed in *S. pombe* is a consequence of low levels of enzyme expression, or, alternatively, of an intrinsically poor activity of the protein. It is possible that our and others inability to detect its activity in cell free assays could be a consequence of a significantly high K_m value for the substrate used ($Man_9GlcNAc$).

It may be speculated that the sharp differences observed between *S. pombe* and *S. cerevisiae* capacities for $Man_9GlcNAc_2$ processing might reflect the absence of a glycan- and proteasome-dependent ERAD pathway in the former yeast. This happened not to be the case because *S. cerevisiae* CPY* expressed in wild-type *S. pombe* cells was degraded to the same extent as in the budding yeast after a 30-min period (Jakob *et al*., 1998). Ablation of genes coding for α-mannosidase or for the mannosidase-like protein (Htm1p/Mnl1p/EDEM) (*spmns1*$^+$ or *spmnl1*$^+$ genes, respectively) resulted, the same as in *S. cerevisiae*, in a drastic reduction in CPY* degradation. This last process was also hindered upon addition of the α-mannosidase inhibitor KFN or of proteasome inhibitors lactacystin and MG132 but not of DMJ or DTT. The differential effects of KFN and DMJ agree with their differential capacities to inhibit in vivo Spmns1p activity. As mentioned above, penetration of DMJ into *S. pombe* ER is probably severely hindered. DMJ at 1 mM concentration, that is, a concentration 50-fold higher than the IC_{50} of the drug for the mammalian cell ER α-mannosidase I, effectively delayed misfolded glycoprotein degradation in those cells (Tremblay and Herscovics, 1999; Tokunaga *et al*., 2000; Wilson *et al*., 2000). This strongly suggests a facilitated penetration of DMJ into the mammalian cell ER lumen.

It is evident that M8B cannot be per se a signal for diverting misfolded glycoproteins to proteasomal degradation in *S. cerevisiae*. Although it is by far the main glycan produced in the ER in this yeast, all glycans transferred to newly synthesized polypeptide chains are converted to it in the ER in all glycoproteins, even in those that fold properly (Byrd *et al*., 1982). That is, no discrimination between properly folded, irreparably misfolded, and folding intermediates can be made by cells based solely on the presence of M8B. Moreover, according to results shown in Figure 8B, demannosylation of $Man_9GlcNAc_2$ presumably occurs immediately after glycan transfer, before glycoproteins complete their folding attempts, that is, before cells have to decide whether to derive glycoproteins to the Golgi or to proteasomes. M8B is the glycan present in *S. cerevisiae* ER α-mannosidase, an ER-permanent resident protein (Vallée *et al*., 2000b), thus indicating that glycoproteins bearing that glycan are not necessarily bound to degradation.

The role of M8B as a signal for protein degradation is even more controversial in mammalian cells. As mentioned above, the main evidence for such a role comes from the observation that α-mannosidase inhibitors such as KFN or DMJ also inhibited misfolded glycoprotein degradation (Liu *et al*., 1997; De Virgilio *et al*., 1999; Chung *et al*., 2000; Marcus and Perlmutter, 2000; Fagioli and Sitia, 2001). Contrary to what happens in both *S. cerevisiae* and *S. pombe*, where there is a single α-mannosidase in the ER and none in the Golgi, two such activities, referred to as I and II, that produce M8B and $Man_8GlcNAc_2$ isomer C (M8C, Figure 1), respectively, have been described in the mammalian cell ER (Weng and Spiro, 1993; González *et al*., 1999; Tremblay *et al*., 1999). In addition, three other α-mannosidases localize to the *cis*-Golgi (referred to as IA, IB, and IC). These three α-mannosidases are able to degrade $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ and are inhibited by both KFN and DMJ. Two of them (IA and IC) yield M8A as first degradation product, whereas IB yields both M8A and M8C (Figure 1) (Lal *et al*., 1998; Tremblay and Herscovics, 2000). The activities of *cis*-Golgi mannosidases are noteworthy because misfolded glycoproteins may cycle between the Golgi and the ER before being diverted to proteasomes (Caldwell *et al*., 2001; Sato *et al*., 2001). In addition, an endomannosidase (not inhibited by KFN or DMJ) present in both the *cis* Golgi and in the ER-Golgi intermediate compartment (ERGIC) may degrade $Glc_1Man_9GlcNAc_2$ (the GT reaction product) to M8A (Zuber *et al*., 2000). M8A is unable to be reglucosylated by GT because it lacks Man residue *g* (Figure 1). Because GT, CNX, and CRT have been described to localize not only to the ER but also to the ERGIC (Zuber *et al*., 2001), endomannosidase degradation of misfolded glycoproteins may trigger their release from the lectin anchors, thus driving them to proteasomal degradation.

The variety of α-mannosidases present in the mammalian cell ER and *cis*-Golgi may explain, for example, that the single *N*-glycan present in 3-hydroxy-3-methylglutaryl-CoA reductase, an ER resident membrane glycoprotein, presents the so-called microheterogeneity because $Man_8GlcNAc_2$, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_5GlcNAc_2$ glycans were detected in the enzyme. M8B and a single $Man_6GlcNAc_2$ isomer were the main species (32 and 43%, respectively) (Bischoff *et al*., 1986). This result confirms that glycoproteins residing in the mammalian cell ER for long periods, as misfolded species do, may have substantial amounts of glycans different from M8B and that those displaying such glycans are not necessarily bound for degradation. Furthermore, it has been determined that glycans in glycoproteins subject to ERAD are processed to $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ (Frenkel *et al*., 2003). Additional observations also cast doubts on the role of M8B as a signal for misfolded glycoprotein degradation in mammalian cells. For example, it was reported that KFN and DMJ delayed proteasomal degradation of a short-lived soluble ribophorin I variant expressed in MadIA214 cells (Ermonval *et al*., 2001). These cells transfer to proteins $Man_5GlcNAc_2$ instead of $Glc_3Man_9GlcNAc_2$ (Figure 1); thus, no M8B can be formed in them. It was also reported that addition of KFN and/or DMJ delayed degradation of misfolded glycoproteins synthesized in the presence of glucosidase I and II inhibitors (deoxynojirimycin and castanospermine) (Tokunaga *et al*., 2000; Wilson *et al*., 2000). Because these compounds prevent removal of glucose units from transferred glycans, no M8B can be formed under the experimental conditions used.

An alternative explanation provided for the role of mannosidases in irreparably misfolded glycoprotein disposal assumes that, as the extensive glycan processing observed in misfolded glycoproteins (formation of $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$) removes the residue to which GT adds the Glc unit (residue *g*, Figure 1), ensuing liberation of misfolded glycoproteins from their CNX/CRT anchors would allow their diversion to proteasomes (Frenkel *et al*., 2003). It was recently reported that both *S. cerevisiae* and mammalian ER α-mannosidases I are not as specific as initially thought because they are able to further degrade M8B (Herscovics *et al*., 2002). It was also suggested that the enhanced misfolded glycoprotein degradation observed upon overexpression of mammalian ER mannosidase I in mammalian cells could be a consequence of arresting GT-mediated reglucosylation (Hosokawa *et al*., 2003). This alternative explanation is not applicable to *S. cerevisiae*, a yeast lacking GT, or to *S. pombe*, because the smallest glycan produced in this yeast ($Man_7GlcNAc_2$, Figure 1) still conserved residue *g*. Furthermore, the above-mentioned reports according to which KFN/DMJ inhibited degradation of misfolded glycoproteins synthesized in the presence of glucosidase inhibitors argue against this alternative explanation: as residues *l-n* (Figure 1) block ER α-mannosidase-mediated removal of residue *g*, no effect of KFN/DMJ addition should had been observed.

All reports on this issue, including the present report, may be best explained by assuming that the effect of ER mannosidase I on misfolded glycoprotein degradation is independent of its enzymatic activity, that is, that ER mannosidase I behaves as a lectin binding polymannose glycans of varied structures, and belongs, together with its enzymatically inactive homologue Htm1p/Mnl1p/EDEM, to a transport chain responsible for delivering misfolded glycoproteins to proteasomes. The crystal structure of *S. cerevisiae* ER α-mannosidase revealed that the enzyme catalytic cavity interacted with the glycan of an adjacent enzyme molecule (Vallée *et al*., 2000b). In addition, evidence was presented indicating that KFN and DMJ occupy the same cavity (Vallée *et al*., 2000a). The observed effects of KFN and DMJ on misfolded glycoprotein disposal could be due not to their activities as ER mannosidase inhibitors but, because they are Man homologues, to inhibition of the ER mannosidase or/and Htm1p/Mnl1p/EDEM putative lectin properties. Furthermore, the above-mentioned presence of a variety of different *N*-glycan structures in irreparably misfolded glycoproteins, the degradation of which is inhibited by KFN/DMJ, point not to a restricted but rather to a broad specificity of the putative ER lectins (both α-mannosidase I and Htm1p/Mnl1p/EDEM) involved in glycoprotein degradation.

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